Immunology 1982 **46** 595

Differential effects of azathioprine on T cells regulating murine B-cell function

HÉLÈNE DUCLOS, MARIE-CHRISTINE MAILLOT & P. GALANAUD *Research Unit I.N.S.E.R.M. U 131, 92140 Clamart, France*

Accepted for publication 25 January 1982

Summary. We describe selective effects of azathioprine (Az) on T-cell subpopulations regulating the primary *in vitro* antibody response of mouse spleen cells to the T-independent antigen TNP-polyacrylamide. This response is susceptible to the effect of two kinds of non-specific suppressor cells: (i) spontaneously-induced suppressors, generated after 4–5 days culture in the absence of antigen; (ii) mitogen-induced suppressors, produced after 2 days in culture in the presence of 2 μg/ml of concanavalin A (Con A). Indeed, both these precultured cells lead to a cell dose-dependent suppression of the anti-TNP response when transferred at the initiation of antigen-stimulated fresh cell cultures. T cells are the effectors of both these suppressions and seem to directly suppress the B-cell response. We tested the *in vitro* effect of Az (10^{-1} μg/ml) on the generation of these two sets of suppressors. Whereas that of Con-A-induced suppressors proves to be resistant, that of spontaneously-induced T suppressors is totally prevented by the addition of Az in the preculture medium. Instead, Az treatment allows the manifestation of a spontaneously-induced helper T cell, simultaneously generated, which is able to increase a T-independent antibody response and quite resistant to the *in vitro* effect of Az. Thus, this study demonstrates that different subpopulations of T lymphocytes regulating the B-cell antibody response exhibit a selective sensitivity to Az, implying different cell proliferation requirements and/or different cellular origin.

Correspondence: Dr H. Duclos, Laboratoire I.N.S.E.R.M. U 131, 32, rue des Carnets, 92140 Clamart, France.

0019-2805/82/0700-0595$02.00

INTRODUCTION

The immunosuppressive drug, azathioprine (Az) is routinely used in clinical situations, even though its precise mechanism and the cellular targets for its activity remain elusive. The present study was initiated to explore the effects of Az on various subpopulations of mouse spleen cells involved in the regulation of the B-cell response. Indeed, Az has proved able to enhance some T-dependent antibody responses (Galanaud, Crevon & Dormont, 1975) and to be useful in distinction between different patterns of lymphocyte activation (Galanaud, Crevon, Erard, Wallon & Dormont, 1976).

Non-specific suppressor cells were generated in two experimental models, either spontaneously in unstimulated cultures (Burns, Marrack, Kappler & Janeway, 1975) or upon activation with the T-cell mitogen concanavalin A (Con A) (Dutton, 1975), and they were assayed on the primary *in vitro* antibody response of mouse spleen cells to the T-independent antigen trinitrophenyl-polyacrylamide (TNP-PAA; Feldmann, Greaves, Parker & Rittenberg, 1974; Galanaud *et al.*, 1976). Indeed, we have previously shown that this response can be influenced by unprimed T suppressor cells selectively sensitive to cyclophosphamide (Duclos, Galanaud, Devinsky,

Maillot & Dormont, 1977). We show here that B cells involved in the anti-TNP response are susceptible to both kinds of *in vitro*-induced non-specific T suppressors. In addition, Az has a contrasting effect on these suppressors, preventing the generation of only spontaneously-induced suppressors, and resulting in an Az-resistant, T-cell-mediated helper effect.

MATERIALS AND METHODS

Animals

DBA/2 mice and, in some experiments, inbred heterozygous nu/+ or homozygous nu/nu C57 Bl/6 mice, were used. All the experimental animals were male, 8–12 weeks old and obtained from CNRS, Orléans, France.

Chemicals

Trinitrobenzene sulphonic acid was supplied by Sigma (St Louis, Mo.). Polyacrylamide beads (Bio-Gel P-30, 100–200 mesh) were obtained from Bio-Rad Laboratories (Richmond, Calif.). 2-mercaptoethanol (2-ME) was purchased from Schwarz-Mann (New York, N.Y.). Con A was purchased from Sigma. o-methyl-alpha-D-mannopyranoside (α-MM) was obtained from Industrie Biologique Française (Clichy, France). Sephadex G-10 was obtained from Pharmacia Fine Chemicals Inc. (Uppsala). Silica was a gift from Dr A. Allison. Azathioprine sodium was purchased from Burroughs-Wellcome (London) and used in cultures at 10^{-1} μg/ml final concentration.

Antisera

Purified sheep anti-mouse immunoglobulin (Ig) antibody (SAMIg) was prepared from a sheep anti-mouse Ig antiserum by affinity chromatography on a mouse Ig-Sepharose 4B (Pharmacia Fine Chemicals Inc., Uppsala) immunoadsorbent. Monoclonal anti-Thy-1·2 antibody was supplied by New England Nuclear (Boston, Mass.). Fluorescent anti-mouse Ig rabbit globulins were purchased from Institut Pasteur (Paris).

Antigens

The TNP conjugate of polyacrylamide (TNP-PAA) was prepared and used as already described (Feldmann *et al.*, 1974).

Culture conditions

Spleen cells were cultured according to Mishell & Dutton (1967) in medium containing 5% foetal bovine serum supplied by Gibco (Grand Island, N.Y.). Cultures were set up in triplicate, either at 10^7/ml cell concentration in 1 ml per 35 × 10 mm tissue culture dishes (Falcon 3001) or at 6–8 × 10^6/ml cell concentration in 0·5 ml per 16 mm well of flat bottom tissue culture plates (Costar 3524). In this latter case 2-ME was added to the culture medium at 5×10^{-5} M final concentration. These procedures yielded comparable results. Unstimulated cultures were set up in parallel to measure the background response. Each experimental group included at least three mice, the spleens of which were pooled before culture.

Induction and assay of suppressor cells

Spontaneously-induced suppressor cells were generated by culturing splenocytes in the absence of antigen in standard 3 ml cultures per 60 × 15 mm tissue culture dishes (Falcon 3002). After 4–5 days, cells were harvested, dead cells removed by centrifugation on Lympholyte-M (Cedarlane Laboratories, Hicksville, N.Y.) and the resulting suspensions washed three times in culture medium. Viable cells were then added, at various precultured: responder cell ratios, on day 0 of fresh cell cultures stimulated with TNP-PAA. The IgM antibody response was measured after 5 days in culture. The suppression index corresponds to [1 − (response with precultured cells : response of fresh cells alone at the same overall cellular concentration)] × 100.

Con-A-induced suppressor cells were generated either by direct addition of Con A in cultures or by incubating splenocytes with 2 μg/ml of Con A in standard 3 ml cultures for 48 hr. In this latter case, Con-A-activated cells were collected and washed two times with a 0·1 M α-MM solution and once without α-MM. Control cells were incubated in parallel and received 2 μg/ml Con A at the end of the incubation period. They were then similarly washed. Incubated cells were added on day 0 of TNP-PAA-stimulated fresh cell cultures. The response of fresh responding cells alone was elicited at the same overall cellular concentration as co-cultures. The suppression index corresponds to [1 − (response with Con-A-incubated cells : response with control-incubated cells)] × 100.

Selective enrichment for B- or T-cell populations

Cells precultured without antigen were selectively enriched for T cells (surface immunoglobulin negative cells, sIg^-) or B cells (surface Ig positive cells, sIg^+) by one or two passages over purified sheep anti-mouse Ig

antibody (SAMIg)-coated polystyrene dishes according to Wysocki & Sato (1978). Cell recovery in non-adherent (sIg^-) and in adherent (sIg^+) fractions, respectively, ranged from 30% to 38% and from 5% to 12% of the starting population. sIg^+ cells accounted for less than 3% of the non-adherent fraction, and for more than 95% of the adherent fraction, as assessed by immunofluorescence.

In one experiment, normal spleen cells were selectively depleted of T cells by treatment with a 10^{-2} dilution of monoclonal anti-Thy-1·2 antibody at a cell concentration of 25×10^6/ml, in the presence of a 1:12 dilution of low toxicity rabbit complement (C; Cedarlane Laboratories, Hicksville, N.Y.)., for 45 min at 37°. Dead cells were removed and surviving cells were more than 98% sIg^+ as assessed by immunofluorescence.

Macrophage depletion

Precultured cells were depleted of macrophages by two consecutive passages through Sephadex G-10 columns according to Ly & Mishell (1974). In one experiment, phagocytic cells were functionally removed by the addition of a Silica suspension to the culture medium according to Allison, Harrington & Birbeck (1966).

Assay for plaque forming cells and expression of the results

Day 5 IgM plaque-forming cell (PFC) response in individual cultures was assayed by the Jerne technique in agarose (Industrie Biologique Française, Clichy) toward TNP-sheep red blood cells (TNP-SRBC; Rittenberg & Pratt, 1969) and toward SRBC. The anti-TNP response was evaluated by deducing the background anti-SRBC PFC from the anti-TNP-SRBC PFC. Collected cells were enumerated in a Coulter counter and results expressed as the number of PFC/10^6 collected cells (PFC/10^6). Results are presented as mean ±SE of the responses or of the suppression indices in different experiments. Statistical analysis was performed using variance analysis and, when comparing suppression indexes, by the Student's *t* test.

RESULTS

Con-A-induced T-suppressor cells of the T-independent response are resistant to Az

As shown in Table 1, Con A directly added at the initiation of mouse spleen cell cultures, induced a dose-related suppression of the anti-TNP response and Con-A-activated cells proved to be able to transfer this suppression in a cell dose-dependent fashion.

Con A acted via a T cell to inhibit the B-cell response as shown by comparing the suppressive effect of Con A (2 μg/ml) on the anti-TNP response from DBA/2 and nu/nu mice. The mean suppression indices differed,

Table 1. Con-A-induced suppression of the T-independent response

Addition in cultures	Anti-TNP response (PFC/10^6)‡ in the presence of:		Suppression index‡	No. of experiments
Con A (μg/ml)*	No Con A	Con A		
0·5	6398±1143	4488±832	20±23	4
1	5168±921	2033±797§	54±19	3
2	5846±1189	1380±250§	77±5	4
4	6972±1307	484±204§	91±3	3
Incubated cells†	Control-incubated cells	Con A-incubated cells		
1:10	7928±1176	3808±1436¶	54±11	2
2:10	4839±1098	1707±619§	68±12	6
3:10	7429±1409	1909±553§	75±2	2

* Con A directly added on day 0 of cultures (10^7 cells/ml) stimulated with TNP-PAA.

† Cells incubated at 10^7/ml cell concentration for 48 hours in the absence (control-incubated cells) or in the presence (Con A-incubated cells) of Con A (2 μg/ml) and then added at various incubated to responder cell ratios on day 0 of fresh cell cultures stimulated with TNP-PAA.

‡ Mean ±SE.

§ $p < 0.001$

¶ $p < 0.05$

Table 2. The generation of con-A-induced suppression is resistant to Az

Az treatment of incubated cells*	Anti-TNP response (PFC/10^6)† in the presence of		Suppression index†
	Control-incubated cells	Con-A-incubated cells	
No	6177±1715	1422±612‡	77±7
+	9467±4359	2186±1147‡	77±9

* Cells incubated for 48 hr in the absence (control-incubated cells) or in the presence (con-A-incubated cells) of Con A(2 μg/ml) and with or without Az (10^{-1} μg/ml) and then added at the 2:10 incubated to responder cell ratio on day 0 of fresh cell cultures stimulated with TNP-PAA.

† Mean ±SE in three experiments (response of fresh cells alone: 8620±6074 PFC/10^6).

‡ $P<0.001$.

respectively 70±3 and 25±0·5 ($P<0.01$) in two experiments. Moreover, Con-A-activated (2 μg/ml for 48 hr) cells obtained from nu/+ or nu/nu mice differently suppressed the response of nu/nu assay cultures (at the 2:10 incubated:responder cell ratio), respectively by 64% (819 PFC/10^6 as compared with 2254 PFC/10^6 in the presence of nu/+ control-incubated cells) and by only 27% (2280 PFC/10^6 as compared with 3124 PFC/10^6 in the presence of nu/nu control-incubated cells). These results clearly demonstrate that the Con-A-induced suppression is T-cell-mediated and can occur in the absence of mature T cells in the assay cultures.

We then tested the *in vitro* effect of Az on the generation of this suppression. As shown in Table 2, the same suppression was obtained regardless of the presence of Az in the culture medium during stimulation of suppressor cells with Con A.

Spontaneously-induced suppression of the T-independent response

As shown in Table 3, spleen cells precultured for 4–5 days in the absence of antigen led to a cell dose-dependent suppression of the anti-TNP response of the assay cultures. The suppressive activity was always recovered in the T-cell-enriched (sIg^-) fraction of precultured cells. In order to exclude that macrophages present in the sIg^- population were the effector cells of the suppression, two sets of experiments were

Table 3. Spontaneously-induced suppression of the T-independent response

Nature of the precultured cells added*	Anti-TNP response (PFC/10^6)† in the presence of		Suppression index†	No. of experiments
	No precultured cells	Precultured cells		
Unfractionated 0·1:10	14,811±2584	13,506±1324	2±20	3
Unfractionated 0·3:10	13,969±1539	9549±1481‡	30±12	5
Unfractionated 1:10	9085±1834	4426±1143‡	52±11	9
Unfractionated 5:10	2467±463	732±362‡	70±11	8
sIg^- cells	4120±958	2361±696‡	42±8	8
sIg^+ cells	5014±1230	7596±2765	—	5
G-10 non-adherent cells	3927±584	1014±468‡	77±7	3

* Cells precultured for 4–5 days in the absence of antigen and then added on day 0 of fresh cell cultures stimulated with TNP-PAA, either unfractionated (at various ratios to responder cells) or upon separation procedures (at 1:10 or 5:10 ratios to responder cells): separation into sIg^- (T-enriched) and sIg^+ (B-enriched) fractions over SAMIg-coated dishes or depletion of adherent cells by two passages through Sephadex G-10 columns.

† Mean ±SE.

‡ $P<0.001$.

Table 4. Differential effect of Az on the generation of spontaneously-induced regulatory T cells of the T-independent response

Precultured cells added*	Az treatment of precultured cells	Anti-TNP response†
0	—	2221 ± 645
Unfractionated cells	no	1657 ± 731
	+	5873 ± 1970
sIg^- cells	no	1366 ± 565‡
	+	5354 ± 1152§

* Cells precultured for 4–5 days in the absence of antigen, with or without Az (10^{-1} μg/ml), and then added on day 0 of fresh cell cultures stimulated with TNP-PAA (at 1:10 precultured:responder cell ratio), either unfractionated or T-cell-enriched (sIg^-) by passage over SAMIg-coated dishes.

† Mean ± SE in three experiments.

‡ $P < 0.025$ as compared with line one.

§ $P < 0.001$ as compared with line one.

performed: (i) adherent cell depletion by passage through Sephadex G-10 columns (Table 3); (ii) addition of Silica (1 mg/ml) for the last 48 hr of the preculture period. Silica-treated cells suppressed by 72% whereas control-cells suppressed by 84%.

Az prevents the generation of spontaneously-induced T suppressors, resulting in a helper effect

When Az was present in the preculture medium, it prevented the generation of suppression, which was replaced by an enhancing effect. This effect was apparent when precultured cells were used without separation (Table 4, and four additional experiments) or when sIg^- cells were used (Table 4). Thus, the enhancing effect is T-cell-mediated. We verified that the precultured cells, when washed and recultured with the antigen, were unable to respond and thus did not participate to the response of the assay cultures (results not shown).

DISCUSSION

Within recent years, numerous studies have well established that Az, a widely used immunosuppressive drug, can influence both humoral and cell-mediated immunity (Bach, 1975). With regard to the *in vitro* concentration of Az we used (10^{-1} μg/ml), which is in the range of those probably obtained *in vivo* when it is clinically administered (Shand, 1980), this drug has interesting selective effects when studied in mouse spleen cell cultures. It completely suppresses the B-cell response to T-dependent antigens (Galanaud *et al.*, 1975) and to certain T-independent antigens (Galanaud *et al.*, 1976). However, it does not affect cell proliferation and polyclonal antibody response induced by LPS (Galanaud *et al.*, 1976), thus showing that it is not toxic and does not completely prevent cellular proliferation. In the present study, we demonstrate that Az can selectively interfere with regulatory mechanisms controlling B-cell function.

We present evidence that the primary *in vitro* antibody response to TNP-PAA is susceptible to non-specific suppressor cells generated either spontaneously in culture or upon stimulation with Con A. This confirms the results of previous studies showing that other T-independent responses can be inhibited by the former (Burns *et al.*, 1975) and the latter suppressor cells (Primi, Hammarström & Smith, 1979). We show that the effector cells of both these suppressions are T cells as (i) no suppression is generated with Con A in nude spleen cells as previously reported (Dutton, 1975); (ii) the spontaneously-induced suppression is exclusively transferred by non-adherent, non-phagocytic sIg^- cells, which fits with several previous studies (Burns *et al.*, 1975; Schreier & Lefkovits, 1979). Moreover, we show that, at the effector stage, the target for both these suppressors could be the B cell, as they appear fully competent to suppress the response of spleen cells respectively from nude mice and from DBA/2 mice

after anti-Thy-1·2 plus C-treatment (results not shown). It should be pointed out that we were unable to decrease the response to TNP-PAA by pretreatment of spleen cells with anti-Thy-1·2 plus C, confirming the classical 'T-independent' character of this particulate antigen (Feldmann *et al.*, 1974; Galanaud *et al.*, 1976). However, this may not be absolute with regard to other studies using the same preparation of TNP-PAA in a human system (Delfraissy, Galanaud, Dormont & Wallon, 1977) or other hapten-carrier presentations in mouse cultures (Puré & Vitetta, 1980) which appear somewhat T-dependent. Thus, we cannot exclude that the expression of the suppression may involve some T-macrophage or T–T cell interaction, as T-amplifier cells induced in other T-independent responses may be one intermediate for the suppressor signal (Markham, Reed, Stashak, Prescott, Amsbaugh & Baker, 1977).

We tested the effect of Az at low concentration (10^{-1} μg/ml) on the generation of both these suppressions. Our data clearly show that Az did not prevent that of Con-A-induced suppression. This discrepancy with the results reported by Dimitriu & Fauci (1978) in a human system can be attributed to the higher concentration (1–10 μg/ml) of Az they used. In contrast, the generation of spontaneously-induced suppression was totally prevented by Az. Instead, Az-treated precultured cells significantly increased the response of fresh assay cultures. Both suppressor and helper activities were T-cell-mediated. Although we cannot definitely exclude that both functions could be supported by the same sIg^- population and that Az acted by stimulating the helper activity, the most likely interpretation is that Az selectively eliminates a T-suppressor subpopulation, resulting in the manifestation of a distinct Az-resistant T-helper subset. Indeed, the concomitant generation of non-specific helper and suppressor cells by culture in the absence of antigen has been well documented for T-dependent antibody responses (Schreier & Lefkovits, 1979). We have shown that these cells can also modulate a T-independent response and that, as in the T-dependent response, suppression appears dominant toward the helper effect. Furthermore, although the effect of low Az concentration that we used may not be entirely explained by an anti-proliferative effect as discussed in Bach (1975), our results fit with different proliferative requirements for these two sets of regulatory cells. Indeed, spontaneously-induced suppressors appear radiosensitive and spontaneously-induced helper cells radioresistant (Chan, Kan & Mishell, 1977) Similarly, the generation of Con-A-induced suppression can take place without concomitant requirement for DNA synthesis (Shand, Orme & Ivanyi, 1980). Thus, the differential sensitivity to Az of these regulatory cell subpopulations suggest that their mechanism of induction and in all probability their precursors are different.

ACKNOWLEDGMENTS

This work was supported by grants from I.N.S.E.R.M. and D.G.R.S.T.

REFERENCES

ALLISON A.C., HARINGTON J.S. & BIRBECK M. (1966) An examination of the cytotoxic effects of Silica on macrophages. *J. exp. Med.* **124,** 141.

BACH J.F. (1975) *The mode of action of immunosuppressive agents*, p. 93, North Holland Ed., Amsterdam.

BURNS, F.D., MARRACK, P.C., KAPPLER, J.W. & JANEWAY C.A. (1975) Functional heterogeneity among the T-derived lymphocytes of the mouse. Nature of spontaneously induced suppressor cells. *J. Immunol.* **114,** 1345.

CHAN E.L., KAN P.B. & MISHELL R.I. (1977). Long-term effects of *in vitro* sensitization on immune reactivity of lymphocytes: generation of suppressor and helper T cells. *J. Immunol.* **118,** 1391.

DELFRAISSY J.F., GALANAUD, P., DORMONT J. & WALLON C. (1977) Primary *in vitro* antibody response from human peripheral blood lymphocytes. *J. Immunol.* **118,** 630.

DIMITRIU W. & FAUCI A.S. (1978) Activation of human B lymphocytes. XI. Differential effects of Azathioprine on B lymphocytes and lymphocytes subpopulations regulating B cell function. *J. Immunol.* **121,** 2335.

DUCLOS H., GALANAUD P., DEVINSKY O., MAILLOT M.C. & DORMONT J. (1977) Enhancing effect of low dose cyclophosphamide treatment on the *in vitro* antibody response. *Europ. J. Immunol.* **7,** 679.

DUTTON R.W. (1975) Suppressor T cells. *Transpl. Rev.* **26,** 39.

FELDMANN M., GREAVES M.P., PARKER D.C. & RITTENBERG M.B. (1974) Direct triggering of B lymphocytes by insolubilized antigen. *Europ. J. Immunol.* **4,** 591.

GALANAUD P., CREVON M.C. & DORMONT J. (1975) Effect of Azathioprine on *in vitro* antibody response. Differential effect on B cells involved in thymus-dependent and independent responses. *Clin. exp. Immunol.* **22,** 139.

GALANAUD P., CREVON M.C., ERARD D., WALLON W. & DORMONT J. (1976) Two processes for B-cell triggering by T-independent antigens as evidenced by the effect of Azathioprine. *Cell. Immunol.* **22,** 83.

LY I.A. & MISHELL R.I. (1974) Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. immunol. Meth.* **5,** 239.

MARKHAM R.B., REED H.D., STASHAK P.W., PRESCOTT B, AMSBAUGH D.F. & BAKER P.J. (1977) Effect of Concanavalin A on lymphocyte interactions involved in the

antibody response to type III pneumococcal polysaccharide. II. Ability of suppressor T cells to act on both B cells and amplifier T cells to limit the magnitude of the antibody response. *J. Immunol.* **119,** 1163.

MISHELL R.I. & DUTTON R.W. (1967) Immunization of dissociated spleen cell cultures from normal mice. *J. exp. Med.* **126,** 423.

PRIMI D., HAMMARSTROM L. & SMITH C.I.E. (1979) Regulation of thymus-independent responses by Concanavalin A-activated spleen cells. *Cell. Immunol.* **42,** 90.

PURE E. & VITETTA E. (1980) The murine B cell response to TNP-polyacrylamide beads: the relationship between the epitope density of the antigen and the requirements of T cell help and surface IgD. *J. Immunol.* **125,** 420.

RITTENBERG M.B. & PRATT K.L. (1969) Antitrinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. exp. Biol.* (N.Y.), **132,** 575.

SCHREIER M.H. & LEFKOVITS I. (1979) Induction of suppression and help during *in vitro* immunization of mouse spleen cells. *Immunology*, **36,** 743.

SHAND F.L. (1980). Attentuation of murine graft-versus-host reactivity by Azathioprine. *Transplantation*, **30,** 55.

SHAND F.L., ORME I.M. & IVANYI J. (1980) The induction of suppressor T cells by Concanavalin A is independent of cellular proliferation and protein synthesis. *Scand. J. Immunol.* **12,** 223.

WYSOCKI L.J. & SATO V.L. (1978) 'Planning' for lymphocytes: a method for cell selection. *Proc. natn. Acad. Sci. U.S.A.*, **75,** 2844.